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Surface-Active Agents from Two *Bacillus* Species

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Two *Bacillus* species were studied which produced bioemulsifiers; however, they were distinctly different compounds. *Bacillus* sp. strain IAF 343 produced unusually high yields of extracellular biosurfactant when grown on a medium containing only water-soluble substrates. The yield of 1 g/liter was appreciably better than those of most of the biosurfactants reported previously. This neutral lipid product, unlike most lipid biosurfactants, had significant emulsifying properties. It did not appreciably lower the surface tension of water. On the same medium, *Bacillus cereus* IAF 346 produced a more conventional polysaccharide bioemulsifier, but it also produced a monoglyceride biosurfactant. The bioemulsifier contained substantial amounts of glucosamine and originated as part of the capsule layer. The monoglyceride lowered the surface tension of water to 28 mN/m. It formed a strong association with the polysaccharide, and it was necessary to use ultrafiltration to effect complete separation. The removal of the monoglyceride caused the polysaccharide to precipitate. It is suggested that earlier reports of biopolymers which both stabilized emulsions and lowered surface tension were actually similar aggregates of lipid and bioemulsifier.

Both the lowering of the surface tension of water and the stabilization of oil and water emulsions can be accomplished by using biological materials. A large variety of biosurfactants have been reported (3, 8, 16, 20, 23). In fact, any lipid probably has some degree of surface activity (3; D. G. Cooper and B. G. Goldenberg, Appl. Environ. Microbiol., submitted for publication). Most lipids are minor components of a cell, however. To have commercial potential as a biosurfactant, a lipid must be produced in high yield to reduce cost and must be excreted into the medium to facilitate efficient product recovery (2, 3, 7).

Most of the bioemulsifiers that have been characterized have been polymeric, usually polysaccharides (1, 3, 16, 19, 20, 24; Cooper and Goldenberg, submitted). Several polymeric biosurfactants have been reported to both stabilize emulsions and significantly lower surface tension (8, 17, 20, 22). It is possible, however, that further fractionation of the crude products would isolate different components that are responsible for each activity (17; Cooper and Goldenberg, submitted).

Often, the extracellular surfactant isolated from a hydrocarbon fermentation is a mixture of carboxylic acids and neutral lipids such as esters, alcohols, and glycerides (4, 8, 9, 11, 14–16). In general, the yields have been poor and no one component has predominated. Traditionally, hydrocarbons have been the substrates of choice to produce biosurfactants and bioemulsifiers, as it was assumed that these compounds were produced to aid metabolism of the oil (11–13, 17, 18, 20–23). There have been examples of the use of water-soluble substrates (2, 3, 16; Cooper and Goldenberg, submitted). This is important for the commercialization of biosurfactants. Single-phase fermentations are simpler than two-phase fermentations, and hydrocarbon substrates are unacceptable for many applications.

Bacillus subtilis and other *Bacillus* species are known to produce lipopeptide biosurfactants (2, 3, 8, 20, 23; Cooper and Goldenberg, submitted). Here we report the properties and characterization of other types of surface-active agents produced by two *Bacillus* species.

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MATERIALS AND METHODS

All data reported in this study are from triplicate measurements. Fermentation data are from at least duplicate runs.

Growth studies. The organisms used in this study were strains IAF 343 and IAF 346, which were isolated from an oil sample by M. Sylvestre, Institute Armand Frappier, Laval, Quebec, Canada. These organisms were identified as a Wolf and Barker group II isolate (*Bacillus* sp. strain IAF 343) and a *Bacillus cereus* strain (IAF 346) by the Microbiology Department of the Royal Victoria Hospital, Montreal, Quebec.

The medium used for the growth of both *Bacillus* species contained KH_2PO_4 (0.3%); Na_2HPO_4 (0.6%); $(NH_4)_2SO_4$ (0.1%); sucrose (1.0%); tryptic soy broth (0.4%); yeast extract (0.01%); and trace amounts of sodium EDTA, $FeSO_4$, $CaCO_3$, $MgSO_4$, and $MnSO_4$.

Fermentation studies were carried out in a 2-liter fermentor (Multigen; New Brunswick Scientific Co., Inc., Edison, N.Y.), and a 1.4-liter working volume was used. Agitation was provided by two flat-blade impellers at 600 rpm. Filtered air was introduced through a sparger at 1.4 liter/min. The temperature was maintained at 37°C and the pH at 5.5 for strain IAF 343. The temperature was maintained at 25°C for *B. cereus*.

Biomass determination. Samples were centrifuged (27,000 × *g*) for 10 min. The pellet was suspended in distilled water and recentrifuged. Biomass was determined by weighing after drying at 105°C for 24 h.

Polysaccharide determination. Samples were treated with three volumes of methanol after the biomass was removed. The precipitate was dried at 105°C and weighed.

Surface tension measurement. Surface tension was measured with a Fisher Autotensiomat, which is a modified de Nouy apparatus. Measurements were made on supernatant samples after centrifugation.

Emulsification measurement. Emulsifier activity was measured by adding 6 ml of kerosene to 4 ml of aqueous sample and vortexing at high speed for 2 min. Measurements were made 24 h later. The emulsion index (E_{24}) is the height of the emulsion layer, divided by the total height, multiplied by 100.

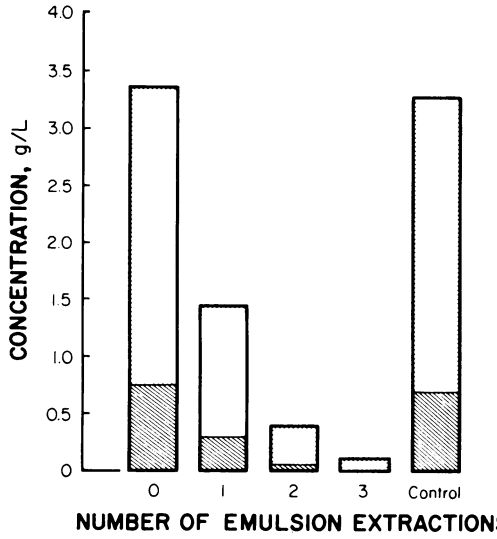

FIG. 1. Analyses of aqueous phase after emulsion extractions. Symbols: dots, carbohydrates; hatches, proteins.

Lipid analyses. The emulsifier was extracted from cell-free medium with chloroform. Thin-layer chromatography (TLC) was conducted with the extracted lipids by using Fisher silica gel (Rediplates GF; Fisher Scientific Co., Pittsburgh, Pa.). The developing solvents were as follows: 1, chloroform-methanol-acetic acid-water (25:15:4:2); 2, hexane-isopropyl ether-methanol (70:30:15); or 3, hexane-isopropyl ether-acetic acid (15:10:1). The standards 1-mono-palmitin, 1,3-dipalmitin, tripalmitin, and palmityl stearate were purchased from Sigma Chemical Co., St. Louis, Mo.

A gas chromatograph (5890; with Hewlett-Packard Co., Palo Alto, Calif.) an integrator (3392; Hewlett-Packard) was used with a capillary column (Durabond Wax; Supelco). The fatty acids were esterified by adding them to 24% tetramethyl ammonium hydroxide. Standard methyl esters and the column were obtained from Supelco.

Emulsifier analysis. Equal volumes of kerosene and cell-free broth were emulsified, and the remaining aqueous phase was extracted. Part of this was analyzed for carbohydrate and protein and the rest was used for successive emulsion extractions. Carbohydrate was measured by the phenol test, and protein was measured by the Coomassie blue reaction (6).

A control experiment involved making the emulsion, breaking this emulsion by centrifuging, and analyzing the aqueous phase.

Polymer analyses. A 30,000-dalton ultrafiltration unit (Millipore Corp., Bedford, Mass.) was used to concentrate crude emulsifier from cell-free, chloroform-extracted broth. The emulsifier was washed with five volumes of distilled water and the precipitate was collected. Samples for high-pressure liquid chromatographic analysis were hydrolyzed in 2 N HCl at 100°C for 2 h. The column used was an Aminex Carbohydrate HPX87C. Standard sugars were obtained from Sigma.

Protein digestion was accomplished with pronase from Boehringer Mannheim Biochemicals, Indianapolis, Ind.). A total of 1 mg of pronase in 5 ml of 0.002 M Tris buffer containing the polymer and a drop of toluene was incubated for 2 days at 37°C and pH 8.2.

Gel electrophoresis was done with a 12% polyacrylamide gel. After developing the gels were stained with Coomassie blue. Standard proteins with molecular weights between 14,000 and 120,000 were obtained from Sigma.

Capsule analysis. The capsule was observed by using wet mounts in India ink. The capsules could be removed by centrifuging the cells at 16,000 × *g* for 15 min. A second treatment was done in 0.1 M phosphate buffer at pH 7.0. A precipitate was obtained by adding three volumes of methanol. This was purified by ultrafiltration.

RESULTS

Surface activity of *B. cereus*. Two days after a sucrose-containing medium was inoculated with *B. cereus*, the surface tension, with or without the cells removed, was lowered to 28 mN/m. The medium was also able to emulsify all the kerosene in the emulsion tests (E_{24}, ca. 60). Again, removal of the cells did not affect this activity.

Extraction of the medium with chloroform did not alter the emulsifying properties but it did remove the surfactant which caused the low surface tension. A single extraction increased this value to 40 mN/m. Successive emulsion extractions of the cell-free broth with kerosene removed both protein and carbohydrate (Fig. 1). A control experiment, in which the emulsion was broken by centrifuging, did not alter the concentration of carbohydrate or protein in the medium, showing that these were not extracted into the oil phase.

Ultrafiltration of the cell-free broth resulted in a filtrate with a low surface tension and no emulsifying capability. Again, the surfactant could be removed from the filtrate by chloroform extraction. The polymer that did not pass through the membrane precipitated but was still a good emulsifier. Recombining the filtrate lipid with the polymer resolubilized the compound.

Electrophoresis of the precipitate revealed more than 10 components. Most of these had molecular weights between 70,000 and 100,000. Samples were incubated with pronase until no protein components remained in the electrophoresis lane. None of these treated polymers showed any loss of emulsifyng activity.

Characterization of lipid from *B. cereus*. The lipid extracted by chloroform from the dialysate was identical to that extracted from the cell-free broth. Results of TLC (Table 1) identified a single component, a monoglyceride. The gas chromatography results (Table 2) showed this to be a mixture of just three homologous lipids which contained only saturated fatty acids. No other peaks were observed.

Characterization of polymer from *B. cereus*. The polymer was not completely characterized, but the high-pressure liquid chromatographic data showed that there was only one major structural unit. After dialysis and hydrolysis, D-

TABLE 1. TLC data for unknowns and standards

Lipid	R_f values for the following solvent mixtures[a]:		
	1	2	3
B. cereus lipid			
Broth	0.95	0.14	0.02
Dialysate	1.0	0.16	0.01
Bacillus sp. strain IAF 343 emulsifier	1.0	0.90	0.83
1-Monopalmitin	1.0	0.11	0.03
1,3-Dipalmitin	1.0	0.44	0.32
Tripalmitin	1.0	0.74	0.70
Palmityl stearate	1.0	0.92	0.80

[a] Solvent mixtures were as follows: 1, chloroform-methanol-acetic acid-water (25:15:4:2); 2, hexane-isopropyl ether-methanol (70:30:15); 3, hexane-isopropyl ether-acetic acid (15:10:1).

glucosamine and very small amounts of two other amino sugars were identified.

The emulsifier was only active below pH 7 (Fig. 2). Between pH 6.5 and 7 there was a very pronounced loss of activity.

Identification of emulsifier from *Bacillus* sp. strain IAF 343. *Bacillus* sp. strain IAF 343 was initially grown for 48 h in shake flasks. At this time the biomass was typically greater than 2 g/liter, and vortexing with kerosene produced stable emulsions. Figure 3 shows the results of the emulsion test performed on shake flask samples after the removal of biomass and then pH adjustment. Between pH 4 and 8 the samples emulsified 60% of the kerosene added. A value of 60% after 24 h compares favorably with values for other emulsifiers reported previously (4–6).

At pH values greater than 9 or less than 3 the emulsion index was halved, and no emulsion formed above pH 11. Samples that were adjusted to above pH 11 and then neutralized did not regain the ability of stabilizing emulsions.

The minimum surface tension obtained for the emulsifier in water was 53 mN/m.

A single extraction of the cell-free broth with chloroform completely removed its ability to emulsify. The lipid residue, after the chloroform was removed, was first analyzed by TLC (Table 1). The solvent mixture 1 moved all of the lipid with the solvent front. This eliminated polar lipids as the active agent. Solvent mixtures 2 and 3 showed that the lipid was a single component and that the R_f values corresponded to those of the ester palmityl stearate that was developed at the same time.

The methyl esters obtained from the unknown ester were compared by gas chromatography with standards. The unknown was a mixture of three saturated carboxylic acids. The position of the three components, their relative compositions, and the position of the appropriate standard are presented in Table 2.

Fermentations. Several fermentations were done with *B. cereus*. Figure 4 shows the results when the pH was maintained at 6.5. The bioemulsifier concentration and E_{24} value were slightly behind the biomass curve. The maximum polymer concentration was 1.6 g/liter. The monoglyceride production was late in the fermentation and the minimum surface tension was not reached until a day after the exponential growth phase was over.

Increasing the pH of the fermentation to 7.0 did not affect monoglyceride production or the concentration of polymer (1.7 g/liter). The broth samples, however, did not stabilize emulsions unless the pH of a sample was lowered from 7.0 to 6.5.

TABLE 2. Relative positions of peaks from gas chromatography for methyl esters of fatty acids

Fatty acid	Relative positions of the following:			Composition (%) in lipid of:	
	Standard	*B. cereus* monoglyceride	*Bacillus* sp. strain IAF 343 ester	*B. cereus*	*Bacillus* sp. strain IAF 343
12:0	3.66				
14:0	4.58		4.65		26
16:0	6.91	6.88	6.97	18	39
18:0	11.32	11.28	11.44	29	35
18:1	12.10				
18:2	17.22				
20:0	19.75	19.72		53	

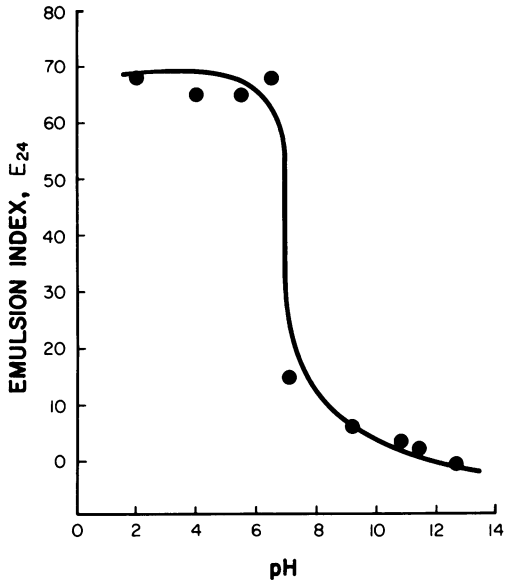

FIG. 2. Effect of pH on the activity of the emulsifier from *B. cereus*.

In a fermentation without pH control the fermentor pH dropped to 5.5 during growth and the yield of polymer was 0.9 g/liter. A fermentation controlled at pH 5.5 yielded 1.0 g/liter.

Cells removed from a fermentor during logarithmic growth and stained with India ink exhibited large capsules around the cells. Cells removed 48 h after inoculation, when the growth was complete, did not have capsules. The capsular material removed from the cells was found to contain appreciable amounts of D-glucosamine.

Figure 5 presents the results of a fermentation of *Bacillus* sp. strain IAF 343 with sucrose at pH 5.5. The fermentation was completed in 25 h. There was no appreciable change in either biomass or lipid concentration after an additional 40 h. The maximum product concentration was 1 g/liter, but

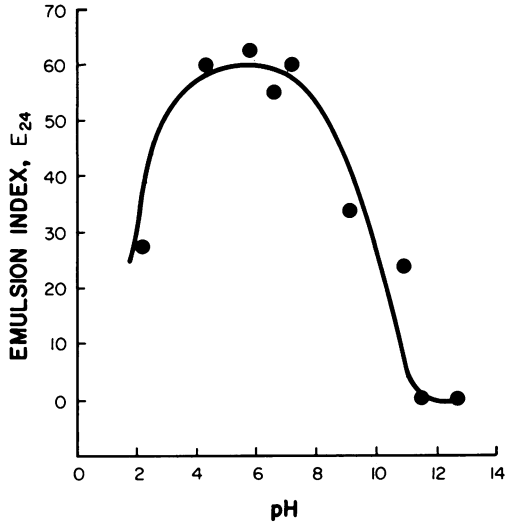

FIG. 3. Effect of pH on the activity of the emulsifier from *Bacillus* sp. strain IAF 343.

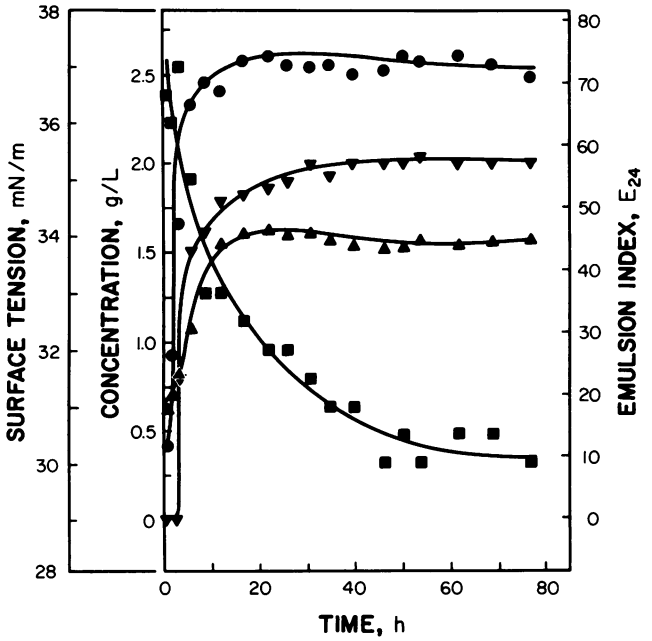

FIG. 4. Batch fermentation of sucrose by *B. cereus* with pH controlled at 6.5. Symbols: ●, biomass; ▲, polysaccharide; ▼, E_{24}; ■, surface tension.

maximum emulsification was obtained with 0.65 g/liter of the lipid. There was little variation in surface tension throughout the fermentation (data not shown).

DISCUSSION

B. cereus produced two surface active agents with very different properties. One was a polymer containing D-glucosamine which stabilized thick oil-in-water emulsions. The other was a homologous mixture of saturated monoglycerides which lowered the surface tension of water to 28 mN/m. Only the monoglycerides were soluble in chloroform, and extraction of the broth with this solvent substantially increased the surface tension. However, some of the lipid was retained in strong association with the polysaccharide. This did not involve a covalent bond because the lipid could be removed by ultrafiltration.

As well as monoglyceride, the crude bioemulsifier also contained a protein associated with the polysaccharide. This protein was extracted into the emulsion interface with the active agent (Fig. 1). Digestion of the crude emulsifier with pronase removed the protein component without decreasing the emulsifying activity. The bioemulsifier emulsan also has an associated protein fraction that is not essential for activity (19, 24). Emulsan is also similar in composition to the *B. cereus* product, as they both contain appreciable amounts of hexosamines.

There are several examples, especially in the earlier studies, of biosurfactants that both lowered surface tension and stabilized emulsions (8, 17, 20, 22; Cooper and Goldenberg, submitted). Usually, these were complex polymeric compounds. A preliminary analysis of the crude emulsifier from *B. cereus* could also lead to the identification of a complex polymer of protein, carbohydrate, and lipid which could both lower surface tension and stabilize emulsions. It seems likely that further characterization of the earlier crude products would uncover polymer bioemulsifiers, lipid biosurfactants, and other fractions without surface activity.

The other *Bacillus* species produced a totally different and unusual type of emulsifier. These bacteria did not cause appreciable reduction of the surface tension of water, but 2 days after inoculation the addition of kerosene to a sample of broth, followed by agitation, resulted in an excellent emulsion. Furthermore, the bioemulsifier was extracellular, and removal of the biomass did not affect the activity.

The ability to stabilize emulsions was eliminated by a single extraction of the cell-free aqueous sample with chloroform. This was unusual because most of the bioemulsifiers produced by microbes are biopolymers, such as that produced by *B. cereus* (3, 16, 20; Cooper and Goldenberg, submitted).

Preliminary analysis of the lipid extract from the cell-free broth by TLC showed that the bioemulsifier was a single neutral lipid, a wax ester. This was also unusual because the surface-active neutral lipids, including esters, are normally isolated as mixtures (4, 9, 11, 14, 15). In these earlier examples, however, the substrate was a hydrocarbon. The mixture of lipids could be the result of indiscriminate extraction of lipids from cellular membranes by the hydrocarbon phase (10, 13; Cooper and Goldenberg, submitted). This extraction mechanism is not operative for a single-phase medium.

All the media used in this study were a single, aqueous phase. It was not necessary to add a water-insoluble oil phase to induce either product. These results support the conclusion that, in general, bioemulsifiers are not produced by microorganisms to facilitate the uptake of an insoluble substrate (3, 19; Cooper and Goldenberg, submitted).

The major effect of hydrocarbons on biosurfactant recovery is probably to extract lipids from the cell membranes (10; Cooper and Goldenberg, submitted). This often results in mixtures of lipids being obtained (4, 9, 14). In this study both bacteria yield a single type of extracellular lipid. Furthermore, the yield of the ester was as much as 1 g/liter. This is significantly greater than the yields of esters obtained from

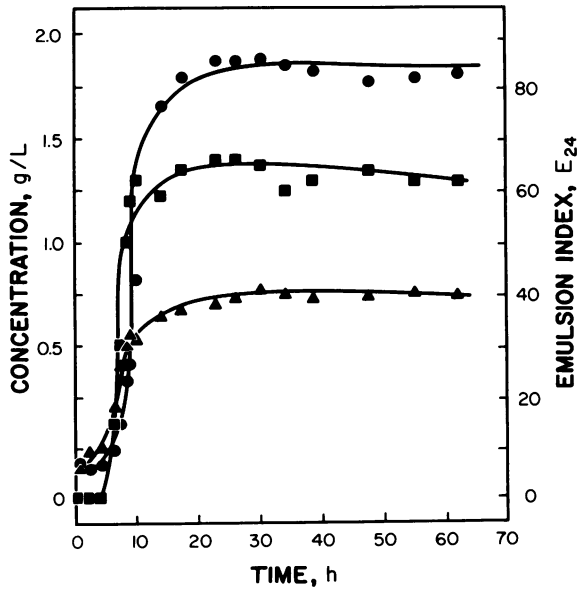

FIG. 5. Batch fermentation of sucrose by *Bacillus* sp. strain 343 with pH controlled at 5.5. Symbols: ●, biomass; ▲, lipid; ■, E_{24}.

the bacteria that metabolize hydrocarbons (11, 14, 15, 21). This *Bacillus* species produced the ester in high yield but not because of the ability of the lipid to act as an emulsifier.

The fermentation data demonstrate that for both bacteria the production of surface-active agents is growth associated, but there are differences between the two microbes. For *Bacillus* sp. strain IAF 343 the strong correlation between lipid concentration and emulsion index confirms that the ester is the only emulsifier produced. There are examples in the literature of biosurfactants that are produced during the exponential growth phase and then decrease in concentration or only appear after growth is completed and cell lysis begins (2, 9). This is not the case for *Bacillus* sp. strain IAF 343, as the concentration of lipid remained constant for several days after growth was complete.

B. cereus is an example of a bacterium in which cell lysis is important for production of the surface-active agents. Both the production of the polysaccharide emulsifier and the lipid, which decreases surface tension, increased as the biomass increased. The polysaccharide concentration, however, reached a maximum about 10 h after the biomass concentration, and the minimum surface tension was observed significantly later than this. Cells stained with India ink exhibited extensive capsules if the samples were taken during exponential growth, but this layer was not observed on older cells. Capsular material isolated from the cells contained significant amounts of glucosamine. The bioemulsifier appeared to be a capsular polysaccharide that was released into the culture medium. Several other bioemulsifiers have been shown to have a similar origin (4, 12, 19).

Not only is the *B. cereus* monoglyceride strongly bound by the polymer but it is also essential for its solubility. If the lipid were not present, the bioemulsifier would have been removed from the system with the biomass. This suggests that the addition of the appropriate surfactant may help in the separation of other bioemulsifiers from the biomass.

The activity of the polymeric bioemulsifier dropped dramatically between pH 6.5 and 7 (Fig. 2). This is a typical range for the protonation of a primary amine. The loss of positive charge on the glucosamine monomers, with increasing pH, removes the emulsifier properties of the polymer. There was a strong pH dependence in the ability of the esters to emulsify (Fig. 1). The total loss of this property at extremely basic pH was irreversible. This indicates that the ester bond is hydrolyzed. These examples of pH sensitivity could be useful for applications in which an emulsion must first be formed and later coalesced quickly. For example, after emulsion pipelining of heavy crude oil, it is necessary to separate the oil and water phases before refining.

Although a change in pH affects the properties of the biopolymer from *B. cereus*, it does not reduce the yield. A fermentation controlled at pH 6.5 (Fig. 3) produced 1.6 g of polysaccharide per liter. If the fermentation was carried out at pH 7.0 the yield was 1.7 g/liter, but there was no emulsion activity in the broth samples until the pH was adjusted to 6.5. This is additional proof that *B. cereus* does not produce the polymer because of its emulsifying properties.

Lowering the pH of the medium to below 6.5 decreased the product yield. If the fermentation was done without pH control, the pH dropped and the yield was reduced. The production and properties of the monoglyceride were not sensitive to any of the pH manipulations described above.

The mixture of esters isolated from *Bacillus* sp. strain IAF 343 was a very effective bioemulsifier. It was possible to obtain emulsion indices as high as 60 which corresponded to complete emulsification of the oil phase. However, it had a poor efficiency. A concentration of 0.65 g/liter was necessary to achieve maximum emulsification. Yield is the major factor in determining the cost of a biosurfactant (2, 6, 7). *Bacillus* sp. strain IAF 343 converted 10% of the carbon source into biosurfactant. This conversion surpassed by a few of the microbes that produce extracellular glycolipids, but these have little, if any, emulsifying ability (3, 7, 8, 17).

The monoglyceride was produced in good yield by *B. cereus* and was very effective at lowering the surface tension. Monoglycerides are currently used as surfactants and can be produced more cheaply from vegetable oils than by fermentation techniques. Results of this study have shown, however, that the addition of a surfactant to the emulsifying polysaccharide enhances its solubility. This greatly improves its dispersion in the system to be emulsified. Although the monoglyceride was not an emulsifier, it can improve emulsion stability by reducing interfacial tension between the two phases. Thus, *B. cereus* produces a polyhexosamine emulsifier, a proven class of bioemulsifiers, in conjunction with a biosurfactant which enhances its activity.

ACKNOWLEDGMENT

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